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14. ABSTRACT
KAI1 is a tumor metastases suppressor gene which is capable of blocking the metastatic process without affecting the primary tumorigenesis. Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test this hypothesis, we will examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo (Task 1), and identify specific peptide sequences that activate KAI1 and to assess the efficacy of the peptides on tumor growth in an animal model (Task 2). We have successfully completed Task 1 and published the results during this cycle. Task 2 is currently underway and one year non-cost extension was requested. Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

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Tumor metastases suppressor, prostate cancer, tumorigenesis

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Table of Contents

INTRODUCTION

The KAI1 gene was originally isolated as a prostate-specific tumor metastasis suppressor gene, using the microcell-mediated chromosome transfer method (1). Ample evidence from both clinical data and the results of in vitro as well as animal experiments overwhelmingly support the notion that KAI1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis (2). Based on our preliminary data, we hypothesize that the KAI1 protein on tumor cells interacts with gp-Fy on the endothelial cells, which activates a signal pathway of the KAI1 molecule, and that this activation eventually leads to cell growth arrest of tumor cells. To test our hypothesis, we will examine whether the interaction of KAI1 and gp-Fy leads to suppression of tumor metastasis in vivo (**Task 1**), and identify specific peptide sequences that activate KAI1 and assess the efficacy of the peptides on tumor growth in an animal model (**Task 2**). Our long-term goal is to elucidate the molecular mechanism of tumor suppression by the KAI1 gene and to develop an effective therapeutic method which restores the function of the KAI1 gene in the metastatic tumor cells.

BODY

Task 1-a. (completed)
Prepare endothelial cells from a gp-Fy knockout and wild type mouse and test their ability to bind and kill tumor cells that express KAI1

As reported last year, we have completed this task and published the results in Nature Medicine (Nature Medicine, 12, 933). As shown in Fig. 2 in this article, we have extensively examined the ability of cancer cell to bind to gp-Fy (DARC) on the endothelial cells. The interaction between Kai1 and gp-Fy blocked tumor cell growth and led them to senescence. We also found that the senescence is induced through activation of TBX2 and p21. To further extend our observation, we are currently using a phospho-specific signal array as well as a gene microarray to identify a specific target which ultimately blocks the metastasis process in response to a signal activated by the interaction of gp-Fy and Kai1.

Task1-b. (completed)
Examine the effects of siRNA against the gp-Fy gene on the binding and killing ability of KAI1 to endothelial cells

We have tested synthetic siRNA to block the expression of gp-Fy in endothelial cells. However, the transfection efficiency for primary culture of endothelial cells had been unexpectedly poor even though we have used several different commercial transfection kits. We then switch to an shRNA expression system using adenovirus vector. However, we also encountered technical difficulties in constructing an effective vector, and the efficiency of shRNA expression was very poor. Accordingly, we abandoned this approach and shifted our effort to in vivo experiments using gp-Fy knockout mice. Our results clearly indicate that the knock-out of gp-Fy nullified the anti-metastatic effect of Kai1 in these mice, suggesting that the gpFy-Kai1 interaction indeed essential for tumor growth arrest.

Task 1-c. (completed)
Examine the metastatic ability of a spontaneously developing tumor in gp-Fy knockout mice by constructing a hybrid animal between a transgenic prostate tumor mouse, TRAMP, and the gp-Fy knockout mouse

Because the doing experiments with a spontaneous mouse model takes a time, we switched to a xenograft model using gp-Fy knockout mouse. We examined the degree of tumor metastases of transplanted tumor cells with or without expression of Kai1 in the gp-Fy knockout mice. As our hypothesis predicted, the degree of metastases dramatically reduced when we transplanted tumor cells with Kai1 in the gp-Fy knockout mouse. These results were published during this reporting cycle (Nature Medicine, 12, 933).

Therefore, we consider that Task 1 has been successfully accomplished.

Task 2-a. (completed)
Construct a series of deletions of gp-Fy and test their binding ability to KAI1.

As we reported in the last report, we completed this task. We tested several individual domains as well as serial deletions from the N-terminus of the KAI1 gene against full length gp-Fy target and vice versa. Our results indicate that the first 32 amino acids from the N-terminus of KAI1, spanning the first intracellular and transmembrane domains, are dispensable for the interaction, but the conformation of the protein as a whole may be important as none of the other fragments yielded a positive interaction. On the other hand, deletion of the first extracellular domain of gp-Fy at N-terminus completely abrogated the interaction, suggesting that the N terminus of gp-Fy is essential for binding to KAI1.

Task 2-b.
Screening a phage display library followed by sequencing the interacting clone

This task is still in progress. We have obtained a phage-display library (Biolab) and have screened the library by first adsorbing the phages to Kai-1 negative cell line followed by recovering the phages that did not attach to these cells. The recovered phages were again adsorbed to Kai+ cells, and phages adsorbed to these cells were recovered. By panning these phages, we isolated 24 different phage clones. We did performed several experiments to confirm our results in vitro. However, the results were not conclusive. We then switched our phage-display library to another source and the screening process is currently underway.

Task 2-c.
Synthesize small peptides corresponding to the gp-Fy binding domain and test for their tumor suppressive activity

Based on the results of Task 2a, we synthesized two different peptides that cover the essential 32 amino acids region for the gp-fy-Kai1 interaction. We first tested in vitro to see if these peptides interfere with the interaction of gp-Fy and Kai1. However, we found that these peptides did not

show any effect on tumor cells. Because another group found that an 18-amino acid peptide corresponding to the N-terminal has biological activity, we also tested this peptide. However, again it did not show any growth inhibitory effect on Kai1+ tumor cells. Although we can synthesize various peptides in this region, it is not a cost effective approach. Therefore, we decided to focus our effort on Task 2-c, so that the results of the experiments will provide us with more options for approach.

Task 2-d.
Test the efficacy of the specific peptides in SCID mice model of prostate cancer

We have not pursued this task because we need to wait for the results of Task 2-c.

KEY RESEARCH ACCOMPLISHMENTS

1. We provided convincing evidence to show that Kai1 interacts with gpFy on the endothelial cells both in vitro and in vivo. We also found that this interaction induce cell senescence by activation of TBX and the p21 genes.
2. We found that the interaction of Kai1 and gpFy is essential to block metastasis in vivo.
3. We narrowed down the region of interaction between gpFy and KAI1 in vitro.
4. We were able to publish the results of Task 1 in the high-impact journal, Nature Medicine.

REPORTABLE OUTCOMES

Peer reviewed publications

1. Bandyopadhyay, S., Zhan, R., Chaudhuri, M., Watabe, M., Pai, S.K., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Takano, Y., Saito, K, Pauza, M., Hayashi, S., Wang, Y., Mohinta, S., Mashimo, T., M. Iiizumi, Furuta, E. and Watabe, K. (2006) Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. Nature Medicine. 12, 933-938.
2. Bandyopadhyay, S and Watabe, K. (2007) The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis in "Developments in Metastasis Suppressors" Nova Publishers. In press
3. Iiizumi, M., Bandyopadhyay, S. and Watabe, K. (2007) Interaction of DARC and KAI1: a critical step in metastasis suppression. Cancer Research. In press

Abstract/presentation

1. Watabe, K. (2006) The role of KAI1 and Drg1 in metastases suppression. BenMay symposium. Chicago
2. Watabe, K. (2006) Metastasis suppressor genes, Kai11 and Drg1. Tokyo University, Japan. Invited lecture
3. Watabe, K. (2006) The role of metastases suppressor genes in prostate cancer. Kyoto University, Japan. Invited lecture

Employment

1. Dr. Megumi Iiizumi (Postdoc) has been supported by the current grant.
2. Sonia Mohinta (currently in Ph.D. program) has been partly supported by the current grant.

CONCLUSIONS

We have successfully completed all experiment proposed in Task 1, and the results were published in Nature Medicine. The paper was cited in two review articles in Nature Medicine and in Cancer Cell. We believe that this conceptual breakthrough about the mechanism of metastases suppression opened a new avenue for the tumor metastases research and also provided with a novel approach for the treatment of prostate cancer. We are planning to elucidate the signal mechanism which leads to the cell senescence. Task 2 is still in progress, and we somewhat struggled with the original approaches. However, with a new screening system of a phage-display library, we hope we can identify peptides which mimic the function of gp-Fy.

So what?
We believe that the progress we have made so far presents a breakthrough concept in the field of tumor metastases. The fact that the interaction between Kai1 and gpFy results in cell senescence through the activation of p21 and TBX provides a bases of new target-specific approach to treat prostate cancer. Our current effort focuses on the development of a method to induce cell senescence through the Kai1 signaling.

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2. Yoshida, B.A., Sokoloff, M.M., Welch, D.R. & Rinker-Schaeffer, C.W. Metastasis-suppressor genes: a review and perspective on an emerging field. *J Natl Cancer Inst.* 92,1717-1730 (2000).

nature medicine

LETTERS

Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression

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***CD82*, also known as *KAI1*, was recently identified as a prostate cancer metastasis suppressor gene on human chromosome 11p1.2 (ref. 1). The product of *CD82* is KAI1, a 40- to 75-kDa tetraspanin cell-surface protein also known as the leukocyte cell-surface marker CD82 (refs. 1,2). Downregulation of KAI1 has been found to be clinically associated with metastatic progression in a variety of cancers, whereas overexpression of *CD82* specifically suppresses tumor metastasis in various animal models[3]. To define the mechanism of action of KAI1, we used a yeast two-hybrid screen and identified an endothelial cell-surface protein, DARC (also known as gp-Fy), as an interacting partner of KAI1. Our results indicate that the cancer cells expressing KAI1 attach to vascular endothelial cells through direct interaction between KAI1 and DARC, and that this interaction leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of TBX2 and p21. Furthermore, the metastasis-suppression activity of KAI1 was significantly compromised in DARC knockout mice, whereas KAI1 completely abrogated pulmonary metastasis in wild-type and heterozygous littermates. These results provide direct evidence that DARC is essential for the function of *CD82* as a suppressor of metastasis.**

We screened the human normal prostate cDNA library using the full-length *CD82* cDNA as bait in a yeast two-hybrid interaction trap[4] and identified Duffy antigen receptor for chemokines (DARC, also known as gp-Fy and encoded by *DARC*) as a potential interactor for KAI1. A liquid β-galactosidase assay quantitatively showed the strength and specificity of the interaction between KAI1 and DARC (**Fig. 1a**). DARC is an approximately 45-kDa, seven-transmembrane protein expressed on vascular endothelium of various organs, as well as on red blood cells and certain epithelial cells[5,6]. It binds chemokines of both C-C and C-X-C families, although ligand binding by DARC does not induce G-protein-coupled signal transduction or Ca^{2+} flux[7,8]. The *DARC* gene has two alleles, Fya and Fyb, which differ only at amino acid residue 44 (ref. 9). Sequence analysis showed that the cloned DNA identified by our screening represents the spliced isoform of the Fyb allele of *DARC* (**Fig. 1b**). To examine the interaction of KAI1 and DARC in mammalian cells, we carried out a coimmunoprecipitation experiment using a highly metastatic prostate carcinoma cell line, AT6.1, which was stably transfected with a Flag epitope–tagged *DARC* gene. The cells were then transiently transfected with a hemagglutinin (HA)-tagged KAI1 plasmid, and the cell lysate was incubated with antibody to Flag. We found that KAI1 coprecipitated with Flag-tagged DARC, suggesting that KAI1 can interact with DARC in mammalian cells (**Fig. 1c**). To localize the regions of KAI1 and DARC that are essential for this interaction, we tested individual domains as well as serial deletions from the amino terminus of KAI1 against full-length DARC target and vice versa in yeast mating assay. Our results indicate that the first intracellular and transmembrane domains of KAI1 are dispensable for this interaction (**Fig. 1d**). On the other hand, deletion of the first extracellular domain of DARC at the amino terminus completely abrogated the interaction, suggesting that the amino terminus of DARC is essential for binding to KAI1 (data not shown).

To assess the relevance of the interaction between KAI1 and DARC, we next examined the localization of DARC in prostate cancer tissue by immunohistochemistry. We found that DARC is highly expressed in the prostate endothelium, particularly in the small veins and venules, as well as in lymphatic vessels, whereas it was undetectable in the epithelial cells and stroma (**Fig. 1e**). The expression of DARC in endothelium was found to be essentially the same in normal, hyperplastic glands and high-grade carcinomas. We observed a similar pattern of expression of DARC in breast and lung cancer samples (data not shown). On the other hand, KAI1 is highly expressed in the normal epithelial cells in these organs, and its expression is substantially reduced in carcinoma, as reported previously[3]. Because expression of *DARC* in these organs is restricted to the vasculature, it is unlikely that KAI1 on epithelial cells interacts with DARC protein in the same cell. Instead, it suggests that such an interaction takes place when cancer cells expressing KAI1 intravasate and encounter the endothelial lining of small blood vessels. Consistent with this hypothesis, a previous study using epifluorescence microscopy detected

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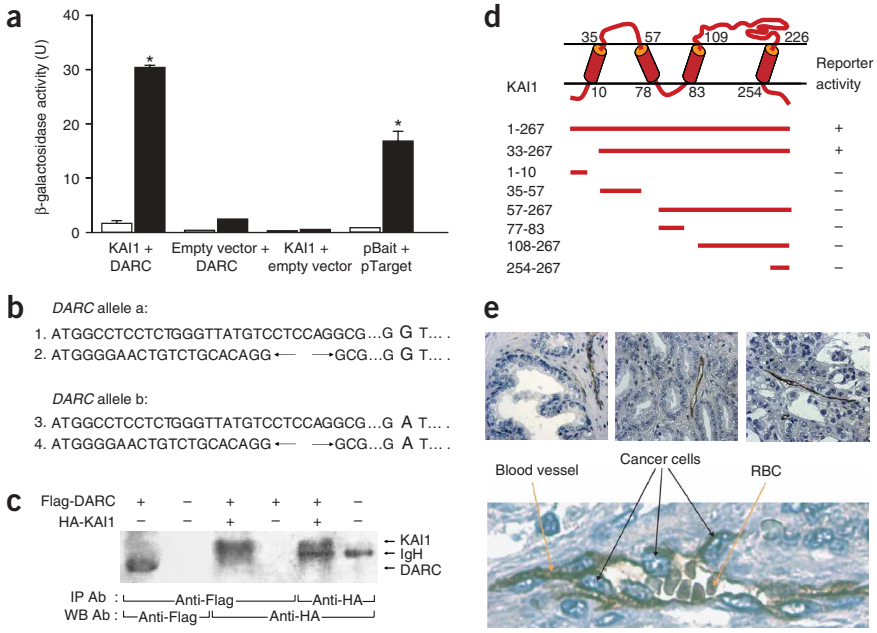

Figure 1 KAI1 interacts with DARC *in vitro*. (**a**) Quantification of interaction between KAI1 and DARC. Yeast cells transformed with an appropriate combination of expression plasmids were grown in minimal medium in the presence of glucose (white bar) or galactose (black bar) as indicated. The β-galactosidase activity is expressed in Miller units (U). pBait and pTarget are a pair of positive control interactors provided by the manufacturer. (**b**) Alleles and splice variants of *DARC*. The junctions of two exons in the biexonic isoforms (#2, #4) are indicated by arrows. (**c**) Coimmunoprecipitation of DARC and KAI1 in mammalian cells. AT6.1/Flag-DARC permanent clone or the parental cell line was tested for DARC expression by immunoprecipitation with monoclonal antibody to Flag covalently crosslinked to agarose beads followed by western blot with monoclonal antibody to Flag (lanes 1, 2). For coimmunoprecipitation, AT6.1/Flag-DARC cells were transiently transfected with HA-tagged KAI1 expression plasmid, proteins were pulled down by Flag-specific agarose beads and KAI1 was detected by western blot with antibody to hemagglutinin (lane 3). To confirm the HA-KAI1 position, the AT6.1/Flag-DARC cells were transfected with HA-KAI1 as above and immunoprecipitation and western bolt were performed with monoclonal antibody to hemagglutinin and protein G agarose followed by western blot with the same monoclonal antibody (lane 5). AT6.1/Flag-DARC cells without KAI1 transfection or parental AT6.1 cells served as negative controls (lanes 4, 6). IgH appeared in lanes 5 and 6, as antibody to hemagglutinin was not crosslinked to the agarose beads during immunoprecipitation. (**d**) Analysis of interactions of various domains of KAI1 with DARC. Regions of KAI1, as indicated by the amino acid sequence numbers, were tested: '+' indicates positive interaction and '–' indicates lack of interaction. (**e**) DARC is expressed only in the vascular endothelium of prostate tissue. Immunohistochemistry was performed on clinical samples using the polyclonal antibody to DARC. Representative fields of normal prostate gland and various grades of prostate carcinoma are shown in the upper panel. DARC is detectable only in the vascular endothelium and red blood cells (RBC). The lower panel represents a magnified view of a blood vessel from a high-grade cancer section.

metastatic tumor cells attached to the endothelium of precapillary arterioles and capillaries in intact mouse lungs[10]. In agreement with this observation, in our archive of specimens, examination of small blood vessels in a high-grade cancer area indicated that cancer cells are often attached to endothelium of blood vessels (**Fig. 1e**).

We next tested the possibility that KAI1 on tumor cells interacts with DARC on endothelial cells by performing a cell-to-cell binding assay *in vitro* in which green fluorescent protein (GFP)-tagged AT6.1 ($KAI1^-$) or AT6.1/Flag-KAI1 ($KAI1^+$) cells were overlaid on $DARC^+$ endothelial cells, human bone marrow endothelial cells (HBMEs) and human umbilical vein endothelial cells (HUVECs). We observed a significantly higher percentage of attachment of $KAI1^+$ cells compared with $KAI1^-$ cells to both types of endothelial cells in a time-dependent manner. Moreover, antibody to KAI1 abrogated this binding, indicating the direct involvement of KAI1 in the process (**Fig. 2a**). We next carried out the same binding assay by overlaying the tumor cells on AT6.1 cells with or without expression of DARC. $KAI1^+$ tumor cells exhibited a binding affinity specifically to the $DARC^+$ AT6.1/Flag-Fy cells (**Fig. 2a**), confirming that the binding of $KAI1^+$ cells to these endothelial cells is indeed due to the expression of DARC. To show a direct interaction between these two membrane proteins in a cell-to-cell manner, we mixed the $KAI1^+$ tumor cells HT-38 and $DARC^+$ HUVECs in the presence of the membrane-impermeable cross-linker 3,3′-dithiobis(sulfosuccinimidylpropionate) (DTSSP), lysed the cells and performed a coimmunoprecipitation experiment. KAI1 coprecipitated with DARC (**Fig. 2b**), whereas another tetraspanin (CD81) did not, indicating a specific interaction between KAI1 and DARC. These results indicate that KAI1-expressing tumor cells can bind to endothelial cells via the interaction between KAI1 and DARC, and suggest the possibility that the metastasis suppressor function of KAI1 is partly due to the trapping of the tumor cells on the endothelial linings of vessels.

It was previously reported that treatment of Jurkat cells with a monoclonal antibody to KAI1 inhibited proliferation of the cells *in vitro*[11]. Therefore, we sought to determine whether this antibody would elicit a similar response in tumor cells expressing KAI1. We found that this antibody significantly inhibited DNA synthesis in $KAI1^+$ prostate tumor cells (**Fig. 2c**). We also obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown). These results suggest that the growth of KAI1-expressing tumor cells is suppressed when KAI1 on the tumor cell surface is engaged by an appropriate ligand. Consistent with this idea, it was previously reported that exposure of prostate tumor cells to nerve growth factor led to upregulation of KAI1, which was also associated with downregulation of cell proliferation *in vitro*[12]. To examine whether the signaling pathway leading to growth arrest of tumor cells is also activated when KAI1 binds to DARC, we measured the rate of DNA synthesis in tumor cells when they were allowed to contact cells that either did or did not express DARC. The rate of DNA synthesis was significantly reduced only when the cells expressing KAI1 (AT6.1/Flag-KAI1) contacted the $DARC^+$ endothelial cells (HBMEs or HUVECs) or the prostate carcinoma cell line (AT6.1/Flag-DARC; **Fig. 2d**). We obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown).

To further corroborate the notion of growth arrest of tumor cells upon interaction with DARC on the endothelial cell surface, we mixed GFP-tagged AT6.1 and AT6.1/Flag-KAI1 cells with HBMEs or HUVECs and then selected for GFP^+ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when

AT6.1/Flag-KAI1 cells (KAI1$^+$), compared with AT6.1 cells (KAI1$^-$), interacted with HBMEs or HUVECs (**Fig. 2e**). We confirmed that this effect is mediated by DARC in the endothelial cells by performing similar experiments in which AT6.1/Flag-KAI1 or AT6.1 cells were mixed with cells with or without DARC expression (AT6.1/Flag-DARC or AT6.1; **Fig. 2e**). Therefore, our data suggest that the interaction between KAI1 and DARC leads to a growth-suppressive effect on the KAI1-bearing cell; thus, the status of KAI1 expression on tumor cells has a key role in determining their fate once they intravasate into the blood vessels.

To examine whether the interaction between KAI1 and DARC is essential for the metastasis suppressor function of KAI1 *in vivo*, we used *Darc*$^{-/-}$ mice[13]. We chose the syngenic metastatic tumor cell lines B16BL6 and B16F10 to establish tumors in these mice and generated several KAI1$^+$ clones or empty-vector transfectants in these cells (**Fig. 3a**). We then injected the B16BL6 derivatives subcutaneously into *Darc*$^{-/-}$ mice and heterozygous and wild-type littermates. We found that primary tumors developed in all mice. The growth rate and final volume of tumors did not significantly vary with the KAI1 level in the tumor cells or with DARC status of the mice (**Table 1**). The KAI1$^+$ clones, however, developed significant numbers of pulmonary metastases in *Darc*$^{-/-}$ mice, whereas metastasis was almost completely abrogated when the same clones were injected in the heterozygous and wild-type littermates (**Fig. 3b** and **Table 1**). The tumor cells lacking KAI1 (B16BL6/vector), however, metastasized equally in all three groups of mice. Thus, in the absence of DARC, even the tumor cells expressing large amounts of KAI1 recapitulated the metastatic phenotype of downregulation of *CD82*. To further corroborate the effect of DARC on the metastatic ability of KAI1-bearing cells, we used an experimental metastasis model in which the metastatic cell line B16F10 stably transfected with KAI1 expression plasmid or an empty vector was injected intravenously into *Darc*$^{-/-}$ mice and their control littermates. The KAI1$^+$ clones resulted in a significantly higher number of pulmonary metastases in the DARC knockout mice, whereas the empty vector transfectant metastasized regardless of the DARC status of the host (**Table 1**). These results support our hypothesis that DARC has a crucial role in the metastasis suppressor function of KAI1 *in vivo*.

DARC is known to be a promiscuous chemokine receptor; however, our *in vitro* data indicate that this function of DARC is not likely to

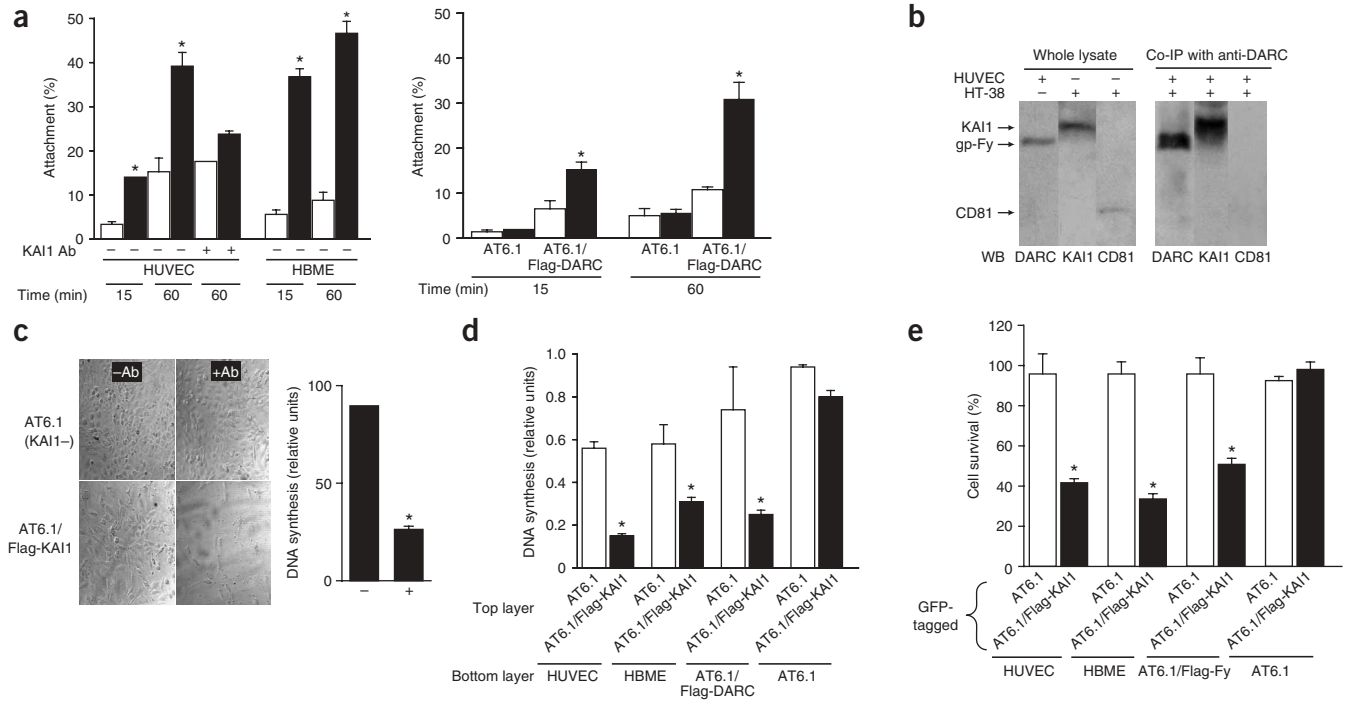

Figure 2 Interaction of KAI1 and DARC leads to growth arrest of cancer cells. (**a**) KAI1 selectively binds to cells expressing DARC in a cell-to-cell binding assay *in vitro*. HBMEs and HUVECs (DARC$^+$; left), AT6.1 and AT6.1/Flag-DARC (right) were grown to confluency. Then, approximately 10^3 cells of AT6.1 tagged with GFP (KAI1$^-$, white bar) or AT6.1/Flag-KAI1 tagged with GFP (KAI1$^+$, black bar) were added on the confluent cell layers, in the presence (+) or absence (–) of monoclonal antibody to KAI1 as indicated. After 15 min or 1 h, wells were washed and the percentage of attachment was calculated as described in Methods. (**b**) Endogenous KAI1 and DARC coimmunoprecipitate in mammalian cells. Lanes 1–3: expression level of KAI1 and CD81 in HT-38 cells and level of DARC in HUVECs were tested by western blot using antibodies to KAI1, CD81 and DARC, respectively. Lanes 4–6: HT-38 and HUVECs were mixed in the presence of a cell-impermeable crosslinker DTSSP for 30 min followed by immunoprecipitation with DARC antibody and western blot with antibodies to KAI1, DARC or CD81, as indicated. (**c**) Monoclonal antibody to KAI1 inhibits growth of KAI1$^+$ prostate epithelial cells. AT6.1 (KAI1$^-$) or AT6.1/Flag-KAI1 (KAI1$^+$) were seeded and monoclonal antibody to KAI1 was added to the wells indicated by '+ Ab' and the rate of DNA synthesis was measured. (**d**) Suppression of DNA synthesis by DARC in prostate cancer cells. DARC$^+$ endothelial cells (HUVECs, HBMEs) and cells with or without DARC expression (AT6.1, AT6.1/Flag-DARC; bottom layer) were grown to full confluency and incubated with 30 μM mitomycin C for 18 h. The cells were then washed extensively, and AT6.1 (white bars) or AT6.1/Flag-KAI1 (black bar) cells (top layer) were added on the monolayer, ^{3}H-thymidine was added to the wells and the incorporation of radioisotopes into DNA of the attached cells was assayed. (**e**) Growth arrest in prostate cancer cells caused by interaction between KAI1 and DARC. Prostate cancer cells expressing both *CD82* and *GFP* genes (AT6.1/Flag-KAI1, black bars) or cells expressing only GFP (AT6.1, white bars) were mixed with cells with or without DARC expression for 1 h followed by plating in the presence of hygromycin, which allowed growth of only GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells. After 5 d, the number of colonies was counted under a fluorescent microscope. *$P < 0.05$.

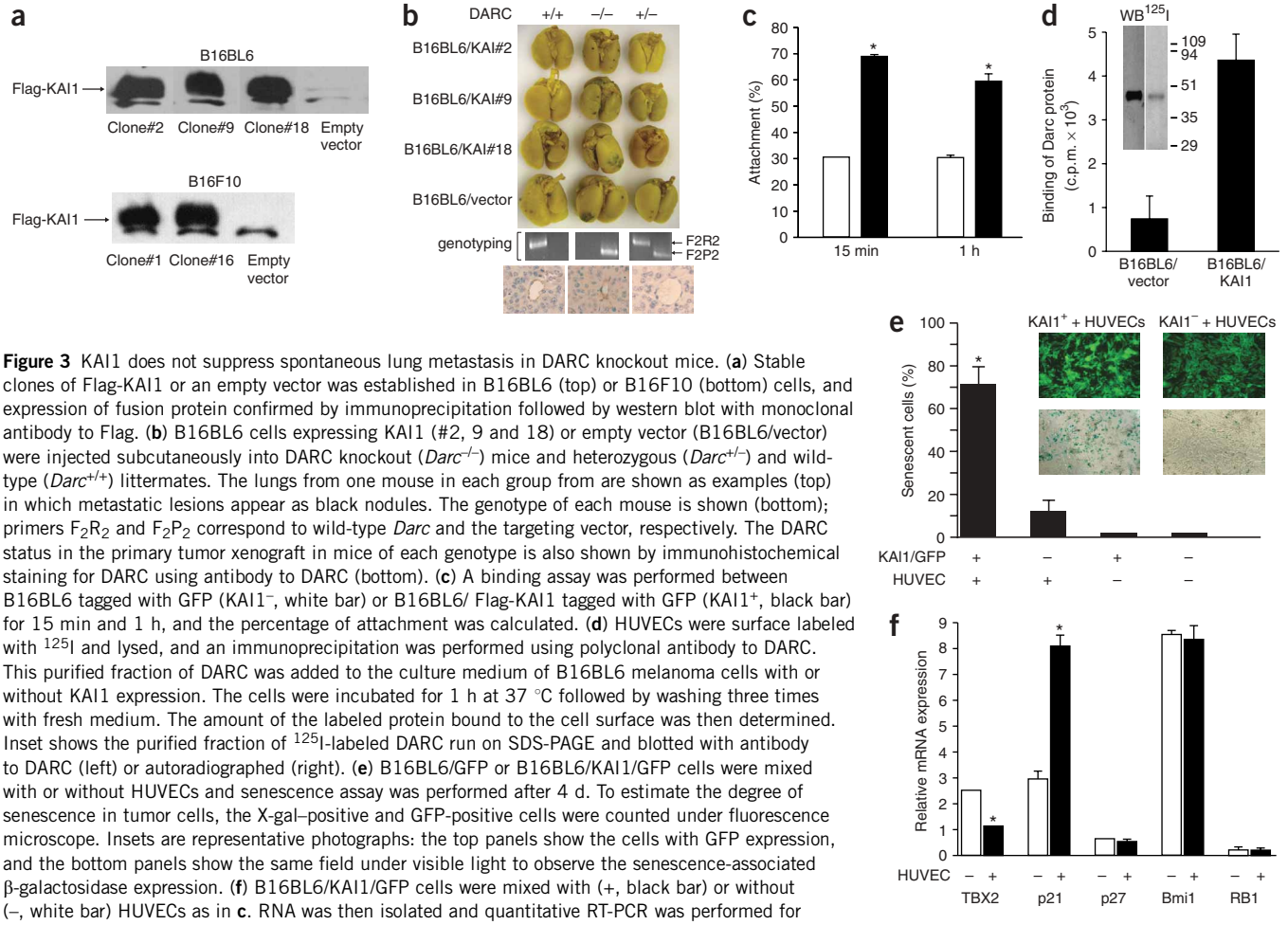

Figure 3 KAI1 does not suppress spontaneous lung metastasis in DARC knockout mice. (**a**) Stable clones of Flag-KAI1 or an empty vector was established in B16BL6 (top) or B16F10 (bottom) cells, and expression of fusion protein confirmed by immunoprecipitation followed by western blot with monoclonal antibody to Flag. (**b**) B16BL6 cells expressing KAI1 (#2, 9 and 18) or empty vector (B16BL6/vector) were injected subcutaneously into DARC knockout (*Darc*$^{-/-}$) mice and heterozygous (*Darc*$^{+/-}$) and wild-type (*Darc*$^{+/+}$) littermates. The lungs from one mouse in each group from are shown as examples (top) in which metastatic lesions appear as black nodules. The genotype of each mouse is shown (bottom); primers F_2R_2 and F_2P_2 correspond to wild-type *Darc* and the targeting vector, respectively. The DARC status in the primary tumor xenograft in mice of each genotype is also shown by immunohistochemical staining for DARC using antibody to DARC (bottom). (**c**) A binding assay was performed between B16BL6 tagged with GFP (KAI1$^-$, white bar) or B16BL6/ Flag-KAI1 tagged with GFP (KAI1$^+$, black bar) for 15 min and 1 h, and the percentage of attachment was calculated. (**d**) HUVECs were surface labeled with ^{125}I and lysed, and an immunoprecipitation was performed using polyclonal antibody to DARC. This purified fraction of DARC was added to the culture medium of B16BL6 melanoma cells with or without KAI1 expression. The cells were incubated for 1 h at 37 °C followed by washing three times with fresh medium. The amount of the labeled protein bound to the cell surface was then determined. Inset shows the purified fraction of ^{125}I-labeled DARC run on SDS-PAGE and blotted with antibody to DARC (left) or autoradiographed (right). (**e**) B16BL6/GFP or B16BL6/KAI1/GFP cells were mixed with or without HUVECs and senescence assay was performed after 4 d. To estimate the degree of senescence in tumor cells, the X-gal–positive and GFP-positive cells were counted under fluorescence microscope. Insets are representative photographs: the top panels show the cells with GFP expression, and the bottom panels show the same field under visible light to observe the senescence-associated β-galactosidase expression. (**f**) B16BL6/KAI1/GFP cells were mixed with (+, black bar) or without (–, white bar) HUVECs as in **c**. RNA was then isolated and quantitative RT-PCR was performed for various senescence-associated genes including *TBX2*, *CDKN1A*, *CDKN1B*, *BMI1* and *RB1*.

have a role in the metastasis-suppression action of KAI1. Rather, DARC seems to directly engage in the interaction with KAI1, which triggers an unknown signal pathway of growth arrest. To obtain mechanistic insight into the interaction between KAI1 and DARC that led to metastasis suppression in our *in vivo* model system, we first carried out a cell-to-cell binding assay using melanoma cells. We found that B16BL6 cells overexpressing KAI1 exhibited a significantly higher binding to the endothelial cells over different time points (**Fig. 3c**), which is consistent with our observation in the case of prostate tumor cells. We then tested the binding of ^{125}I-labeled purified fraction of DARC to the cell surface of B16BL6 melanoma cells with or without KAI1 expression. A significantly ($P < 0.05$) higher amount of DARC bound to the melanoma cells expressing KAI1 compared with the empty vector transfectant (**Fig. 3d**), supporting our notion that KAI1 and DARC interact at the surface of the tumor cells. Such interaction leads to growth arrest of tumor cells (**Fig. 2d,e**). However, we did not detect apoptosis in the KAI1$^+$ tumor cells by TUNEL assay upon coculturing with DARC$^+$ cells (data not shown). We therefore examined whether the interaction with DARC leads to senescence in the KAI1$^+$ tumor cells by mixing HUVECs with GFP-tagged B16BL6 cells with or without KAI1 expression. We found that a significant percentage of KAI1$^+$ tumor cells underwent senescence as a result of interaction with HUVECs (**Fig. 3e**). Furthermore, we found that expression of the senescence-associated gene *TBX2* was reduced and *CDKN1A* (encoding p21) was upregulated in these cells upon interaction with HUVECs, whereas *CDKN1B* (encoding p27), *BMI1* or *RB1* did not show any appreciable change in expression level (**Fig. 3f**). Notably, several previous publications showed a potential link between tumor progression and senescence[14–18]. Particularly consistent with our results, TBX2 has been found to inhibit senescence by directly repressing p21 expression in melanoma cells, suggesting that the TBX2-p21 pathway has a crucial role in tumor progression[19].

Collectively, our results indicate that when tumor cells dislodge from the primary tumor and intravasate into the blood vessels, tumor cells expressing KAI1 attach to the endothelial cell surface, whereby KAI1 interacts with DARC. This interaction transmits a senescent signal to the tumor cells, whereas those that lost KAI1 expression proliferate in the circulation, potentially giving rise to metastases. Notably, KAI1 as a tetraspanin was previously shown to interact with several other cell-surface proteins including α4β1 integrin[20]. The presence of these integrins on tumor cells promotes attachment to vascular endothelial cells[21]. Therefore, the association of integrin and KAI1 may have a part in the KAI1-DARC interaction, although this possibility needs to be explored further. Nonetheless, our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting formation of primary tumors. It highlights a previously unappreciated function of *DARC* and identifies DARC as a new candidate for potential therapeutic intervention for metastatic cancer.

Table 1 Spontaneous and experimental metastases of B16BL6/KAI1 cells in DARC knockout mice

Spontaneous metastases of B16BL6/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Tumor volume (mean ± s.e.m.)			Incidence of pulmonary metastasis			
		Darc$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	*Darc*$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	*P* value
2	Positive	4.9 ± 0.03	4.5 ± 0.02	4.5 ± 0.01	2/15 (13.3%)	9/15 (60%)	1/15 (6.7%)	0.02[a], 0.008[b]
9	Positive	4.6 ± 0.05	4.5 ± 0.03	4.9 ± 0.04	1/15 (6.7%)	6/13 (46.2%)	1/15 (6.7%)	0.05[a], 0.05[b]
18	Positive	4.5 ± 0.05	4.2 ± 0.03	3.9 ± 0.04	0/13 (0%)	6/12 (50%)	0/13 (0%)	0.04[a], 0.04[b]
Empty vector	Negative	4.9 ± 0.05	4.8 ± 0.05	4.9 ± 0.03	6/15 (40%)	5/14 (35.7%)	5/14 (35.7%)	0.8[a], 0.89[b]

Experimental metastases of B16F10/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Number of pulmonary metastases			*P* value
		Darc$^{+/+}$	*Darc*$^{-/-}$	*Darc*$^{+/-}$	
1	Positive	4.7 ± 2.4 ($n = 9$)	47.86 ± 5.9 ($n = 7$)	2.8 ± 0.8 ($n = 6$)	<0.001[a], <0.001[b]
16	Positive	4.4 ± 2.4 ($n = 7$)	32.14 ± 3.6 ($n = 7$)	9.4 ± 2.7 ($n = 5$)	<0.001[a], 0.001[b]
Empty vector	Negative	40.0 ± 8.4 ($n = 5$)	56.0 ± 11.8 ($n = 5$)	32.5 ± 4.8 ($n = 6$)	0.3[a], 0.08[b]

[a]Comparison between *Darc*$^{-/-}$ and *Darc*$^{+/+}$. [b]Comparison between *Darc*$^{-/-}$ and *Darc*$^{+/-}$.

METHODS

Yeast two-hybrid screening. We cloned full-length *KAI1* cDNA cloned into the yeast vector pEG202-NLS (Origene Technologies) as bait, and performed yeast two-hybrid screening and mating assay according to the manufacturer's protocol.

Quantitative β-galactosidase assay. We performed the β-galactosidase assay (Miller test) as previously described[22].

Cell culture. The rat prostatic carcinoma cell line AT6.1, the human breast carcinoma cell line MDA-MB-435, HBMEs and the mouse melanoma cell lines B16BL6 and B16F10 were provided by C. Rinker-Schaeffer (University of Chicago), B.E. Weissman (University of North Carolina at Chapel Hill), K. Pienta (University of Michigan Medical School) and I.J. Fidler (M.D. Anderson Cancer Center), respectively. We purchased the human lung epithelial carcinoma cell line A549 and colon carcinoma cell line HT-38 from American Type Tissue Culture Collection. We cultured the cells in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 250 nM dexamethasone and antibiotics. We obtained HUVECs from Clonetics and cultured them in endothelial growth medium (EGM, Clonetics) as per the manufacturer's instruction.

Immunoprecipitation and western blot. For coimmunoprecipitation experiments using the AT6.1 cells, approximately 48 h after transfection, we harvested cells and lysed them in ice-cold lysis buffer (1% NP40, 10 mM Tris, pH 8.0, 150 mM NaCl, 3 mM $MgCl_2$, 2 mM PMSF) for 45 min and centrifuged them at maximum speed for 15 min. For immunoprecipitation with monoclonal antibody to Flag, we used Flag-specific M2 affinity gel (Sigma). For immunoprecipitation with antibody to hemagglutinin, we incubated the lysate with monoclonal antibody to hemagglutinin (Boehringer Mannheim) and used protein G-Sepharose beads. After immunoprecipitation, we thoroughly washed the beads, and analyzed bound proteins by western blot using monoclonal antibody to hemagglutinin or monoclonal antibody to Flag (Sigma) at dilutions of 1:400 and 1:500, respectively. For coimmunoprecipitation of endogenous KAI1 and DARC, we mixed the KAI1$^+$ tumor cell line HT-38 with DARC$^+$ HUVECs in the presence of the cell-impermeable cross-linker DTSSP for 30 min at 24 °C. We lysed the cells in the same lysis buffer as above, centrifuged them and immunoprecipitated the lysate with rabbit polyclonal antibody to DARC in the presence of protein G agarose beads. After immunoprecipitation, we analyzed bound proteins by western blot using antibody to DARC (1:500), mouse monoclonal antibody to KAI1 (1:1,000, a gift from O. Yoshie, Shionogi Institute for Medical Science) or mouse monoclonal antibody to CD81 (1:20, Chemicon).

Immunohistochemistry. We carried out immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate, breast and lung, using polyclonal antibody to DARC. Briefly, we deparaffinized sections, rehydrated them and heated them at 80 °C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. We treated sections with 3% H_2O_2 to block endogenous peroxidase activity and then incubated them with primary antibody (1:50 dilution) for 1 h at 24 °C. After washing in Tris-buffered saline/0.1% Tween-20, we incubated sections with horseradish peroxidase–conjugated rabbit-specific IgG (Dako Corp.). We washed sections extensively, and applied DAB substrate chromogen solution followed by counterstaining with hematoxylin. The Southern Illinois University Institutional Review Board approved obtaining human specimens for this study.

Cell-to-cell binding assay. We seeded HBMEs, HUVECs, AT6.1 or AT6.1/Flag-DARC (DARC$^+$ permanent clone established in AT6.1) cells in 24-well plates and grew them to full confluency. We trypsinized cells used for overlaying (AT6.1/GFP and AT6.1/Flag-KAI1/GFP, or B16BL6/GFP and B16BL6/Flag-KAI1/GFP) and resuspended them in RPMI medium, and added 10^3 cells on the confluent bottom cell layers in the presence or absence of antibody to KAI1. After 15 min or 1 h, we washed the wells with RPMI medium three times and incubated the cells for 12 h at 37 °C. The numbers of cells attached on confluent monolayers were then counted by observing GFP signal under a confocal microscope and the percentage of attached cells was calculated. For each data point, experiments were performed in triplicate wells and ten random fields were counted in each well.

Treatment of tumor cells with monoclonal antibody to KAI1. We seeded approximately 10^3 cells of AT6.1 and AT6.1/Flag-KAI1 in 96-well plates. We then added ^{3}H-thymidine with or without monoclonal antibody to KAI1 (provided by H. Conjeaud, Cochin Hospital) to the wells, which we then incubated at 37 °C for 48 h. The ^{3}H-thymidine incorporation by the AT6.1/KAI1 cells was normalized with respect to the incorporation by the AT6.1 cells. Each experiment was performed in triplicate.

Measurement of DNA synthesis. We cultured HUVECs, HBMEs, AT6.1 and AT6.1/Flag-DARC cells to confluency and then treated them with mitomycin C for 18 h to block DNA synthesis. After washing the wells extensively with RPMI media, we seeded 10^3 AT6.1 cells that did or did not express KAI1 (AT6.1/Flag-KAI1 or AT6.1) on the monolayer of mitomycin C–treated cells and added ^{3}H-thymidine to the wells. We incubated the cells at 37 °C for 48 h, then washed the wells with RPMI media three times and measured the incorporation of ^{3}H-thymidine in the attached cells. The rate of DNA synthesis by the cells

seeded on monolayers was normalized by that of cells seeded directly on the plastic plate. Each experiment was performed in triplicate.

Colony formation assay. We trypsinized HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells, resuspended them in RPMI medium and mixed them with AT6.1 cells, which expressed the gene encoding GFP with or without KAI1 (AT6.1 or AT6.1/Flag-KAI1, both GFP tagged), for 1 h, then plated the mixture in RPMI medium containing hygromycin. The GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells were also plated without mixing with HUVECs, HBMEs, AT6.1 or AT6.1/DARC cells for the purpose of normalization. We incubated the cells at 37 °C for 5 d and counted the number of colonies expressing GFP under the fluorescence microscope. The number of colonies formed by GFP-tagged AT6.1 or AT6.1/Flag-KAI1 mixed with HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells was normalized with the number of colonies formed by the GFP-tagged cells alone. Each experiment was done in triplicate.

***In vivo* metastasis assay.** For spontaneous metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml of PBS of various B16BL6 clones subcutaneously in the dorsal flank of the DARC knockout mice as well as heterozygous and wild-type littermates. We monitored mice daily for the growth of primary tumor. After 6 weeks, mice were killed, tumor volume was calculated using the equation Volume = (Width + Length)/2 × width × length × 0.5236, and metastatic lesions were counted macroscopically. For experimental metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml PBS of various B16F10 clones intravenously into the tail vein of the DARC knockout mice as well as control littermates. Mice were killed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically. All protocols were approved by the Southern Illinois University Institutional Review Board.

***In vitro* binding assay.** The DARC$^+$ cells were surface labeled with ^{125}I using Iodo-beads (Pierce) according to the manufacturer's protocol. We lysed the cells and immunoprecipitated them using antibody to DARC and protein G agarose. We washed the agarose beads extensively to remove unbound proteins and eluted the bound proteins using 0.1 M glycine, pH 3.5, immediately followed by neutralization with 0.5 M Tris, HCl, pH 7.4, 1.5 M NaCl. This eluate was further concentrated by Centricon P10. B16BL6 cells with or without KAI1 expression were seeded in 24-well plates and grown to confluency. We added the purified protein to the cells in culture and 48 h later, washed the wells three times with fresh medium and determined the amount of the bound protein.

Senescence assay. We trypsinized B16BL6/GFP or B16BL6/Flag-KAI1/GFP cells, resuspended them in medium and mixed them with the DARC$^+$ HUVECs for 1 h followed by plating the mixture. We also plated the GFP-tagged cells without mixing with HUVECs as control. We incubated the cells at 37 °C for 4 d. We then performed a senescence assay using a senescence-associated β-galactosidase detection kit (Calbiochem) according to the manufacturer's instruction, and counted the X-gal–positive and GFP-positive cells under a fluorescence microscope.

Real-time RT-PCR. We mixed B16BL6/Flag-KAI1/GFP cells with or without the DARC$^+$ HUVECs for 1 h, and then plated the mixture and incubated it at 37 °C for 4 d. We isolated total RNA from the cells and reverse-transcribed it. We then amplified the cDNA with a pair of mouse-specific forward and reverse primers for the following genes: *TBX2* (forward, 5′-CACCTTCCGCACCTATGTC-3′; reverse, 5′-CAAACGGAGAGTGGGCAGCGTT-3′), *CDKN1A* (forward, 5′-CCGTGGACAGTGAGCAGTT-3′; reverse, 5′-CCAATCTGCGCTTGGAGTGA-3′), *BMI1* (forward, 5′-AATCCCCACTTAATGTGTGTC-3′; reverse, 5′-TCACCTCTTCCTTAGGCTTCTC-3′), *CDKN1B* (forward, 5′-GTGGACCAAATGCCTGACT-3′; reverse, 5′-GGCGTCTGCTCCACAGTG-3′), *RB1* (forward, 5′-TGATGAAGAGGCAAACGTGG-3′; reverse, 5′-TGGCCCACAGCGTTAGCAAAC-3′) and β-actin. We performed PCR using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR Kit (Finnzyme Corp). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 15 min followed by 30 cycles of PCR using the following profile: 94 °C for 30 s; 57 °C for 30 s; 72 °C for 30 s.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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